A Major T Cell Antigen of *Mycobacterium leprae* Is a 10-kD Heat-shock Cognate Protein

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Summary

Several mycobacterial antigens, identified by monoclonal antibodies and patient sera, have been found to be homologous to stress or heat-shock proteins (hsp) defined in *Escherichia coli* and yeast. A major antigen recognized by most *Mycobacterium leprae*–reactive human T cell lines and cell wall–reactive T cell clones is a 10-kD protein that has now been cloned and sequenced. The predicted amino acid sequence of this protein is 44% homologous to the hsp 10 (GroES) of *E. coli*. The purified native and recombinant 10-kD protein was found to be a stronger stimulator of peripheral blood T cell proliferation than other native and recombinant *M. leprae* proteins tested. The degree of reactivity paralleled the response to intact *M. leprae* throughout the spectrum of leprosy. Limiting-dilution analysis of peripheral blood lymphocytes from a patient contact and a tuberculoid patient indicated that approximately one third of *M. leprae*–reactive T cell precursors responded to the 10-kD antigen. T cell lines derived from lepromin skin tests were strongly responsive to the 10-kD protein. T cell clones reactive to both the purified native and recombinant 10-kD antigens recognized *M. leprae*–specific epitopes as well as epitopes crossreactive with the cognate antigen of *M. tuberculosis*. Further, the purified hsp 10 elicited strong delayed-type hypersensitivity reactions in guinea pigs sensitized to *M. leprae*. The strong T cell responses against the *M. leprae* 10-kD protein suggest a role for this heat-shock cognate protein in the protective/resistant responses to infection.

Understanding resistance to leprosy and other infectious diseases caused by intracellular pathogens requires identification of antigens that stimulate cell-mediated immune responses. The fact that *Mycobacterium leprae* remains one of the few bacterial pathogens of humans that has not been successfully cultivated in vitro poses a serious challenge to the identification and purification of protective antigens. Several biochemical, immunological, and molecular approaches have recently been used for identification and characterization of protein antigens of the leprosy bacillus (1–6). Over 10 antigens have been identified and cloned by use of monoclonal antibodies or patient sera. Of these, the 70-, 65-, 36-, 35-, 18-, and 17-kD antigens elicit T cell reactivity in a small number of sensitized individuals, although it remains unclear which, if any of them, has a significant role in protective immunity (7–12). Four of these mycobacterial antigens share significant homology with highly conserved heat-shock proteins (hsp)[1] or stress proteins in *Escherichia coli*, yeast, and other organisms (6).

A number of new strategies have been developed for use of T cells to screen and identify mycobacterial antigens that may be involved in protective immune responses. T cell clones have been used directly to screen pools of phage clones from the recombinant λgt 11 library of *M. leprae* (13). Several groups

[1] *Abbreviations used in this paper:* hsp, heat-shock protein; DTH, delayed-type hypersensitivity; IPTG, isopropylthiogalactoside; FPLC, fast protein liquid chromatography; MBP, maltose-binding protein.

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have used the T cell Western blot approach, where PBMC, T cell lines, or clones are employed to screen proteins of *M. leprae* separated by SDS-PAGE and blotted onto nitrocellulose membranes for analysis (4, 14–17). Previous studies of *M. leprae* antigens from our laboratories using the T cell Western blots indicated that most T cell lines from reactive donors recognized an antigen in the molecular mass range of 7–10 kD (4). We now report the isolation, sequencing, and expression of the gene encoding this 10-kD protein as well as our evaluation of the ability of purified native and recombinant protein to stimulate T cell responses in reactive individuals in vitro and specific delayed-type hypersensitivity (DTH) responses in vivo.

Materials and Methods

Patients. Patients with leprosy were diagnosed at the Los Angeles County Hansen's Disease Clinic (Los Angeles, CA) and the Institute of Biomedicine (Caracas, Venezuela). They were classified according to the criteria of Ridley and Jopling (18).

Some patients with tuberculoid leprosy were skin tested with 0.1 ml of lepromin A (armadillo derived, cobalt-irradiated, 2×10^6 bacilli/ml) given intradermally. Punch biopsy was performed at 21 d on reactions with an induration of ≥5 mm in diameter.

Antigens. Armadillo-derived *M. leprae* was obtained from R. J. W. Rees through the IMMLEP Program, WHO (Geneva, Switzerland). The biochemical purification of the native proteins of *M. leprae* used in this study has been extensively described (5). These were the 10-kD (previously known as a 14-kD protein), the 3-, 17-, 22-, 28-, and 35-kD proteins. The 10-kD protein of *Mycobacterium tuberculosis* (Erdman) was purified as described for the *M. leprae* product. Purified recombinant 65-kD and 70-kD proteins were kindly provided by Dr. Van Embden (National Institute of Public Health, Bilthoven, Netherlands), and the 18-kD protein was supplied by Dr. J. Watson (University of Auckland, New Zealand). The 27-mer representing the NH_2-terminal sequence of the *M. leprae* 10-kD protein was synthesized on a peptide synthesizer (430A; Applied Biosystems, Inc., Foster City, CA) and purified by reverse phase HPLC. Overlapping peptides of *M. tuberculosis* 10-kD antigen were a generous gift from Dr. Percy Minden (Scripps Clinic and Research Foundation, La Jolla, CA). Tetanus toxoid was obtained from the Massachusetts Department of Health (Boston, MA). The concentration of antigens and peptides used was determined by several titration experiments on responsive cells.

T Cell Western Blot. Immunoreactive *M. leprae* antigens were identified by the T cell Western blot method (4). Briefly, *M. leprae* sonicate (4 mg/ml) was subjected to electrophoresis on 5–20% gradient SDS-PAGE gel to separate the proteins. After electrophoresis proteins were transferred onto nitrocellulose membrane, cut into 2 × 50-mm strips, dissolved in DMSO, and reprecipitated by the addition of 0.05 M bicarbonate buffer (pH 9.6) to convert nitrocellulose into fine antigen-bearing particles. The precipitate was washed with PBS and resuspended in 0.6 ml of RPMI. For lymphoproliferative assays, 10 μl of the suspension was added to cultures.

Isolation and NH_2-terminal Sequence of 10 kD Protein. The 7–10-kD major T cell–stimulating protein was isolated by the following procedure. A T cell Western blot was performed on *M. leprae* proteins separated by SDS-PAGE using part of the gel (three lanes), while the remaining gel was saved for elution of relevant proteins. The proteins were electroeluted (Extraphor Electrophoretic Concentrator; LKB Instruments, Inc., Bromma, Sweden) from the area of polyacrylamide gels corresponding to the Western blot nitrocellulose strips that induced proliferation of the T cell clones/lines previously reactive to a protein in this molecular mass range. By use of this procedure, 7–10-kD molecular mass range proteins were pooled from four gels and concentrated with a microconcentrator centricon-3 (Amicon, Beverly, MA). The polypeptides were resolved further by two-dimensional PAGE; two-dimensional gels were performed by Protein Databases, Inc. (Brookhaven, NY) using published methods (19). After electrophoresis the proteins were visualized with silver stain.

Proteins from an identical preparative two-dimensional gel were transferred onto an Immobilon (Millipore, Bedford, MA) membrane, visualized with coomassie blue, and both the protein spots were directly subjected to microsequencing at the University of Wisconsin Biotechnology Center (Madison, WI). The sequence of 24 NH_2-terminal amino acids of each spot was determined from 15 pmol of protein.

Screening of λgt 11 M. leprae Library. The gene encoding the 10-kD protein of *M. leprae* was cloned by use of synthetic oligonucleotide probes to screen the λgt 11 library of *M. leprae* genomic DNA. Initially these probes were designed to correspond with the amino acid sequence of the NH_2 terminus, based on the most frequently used codons reported in the nucleotide sequences of the genes encoding mycobacterial proteins. Subsequent screening was performed using probes designed to be homologous to the 5′-nucleotide sequence of the bacille Calmette-Guerin (BCG) protein of *M. tuberculosis*.

The recombinant λgt 11 library of *M. leprae* genomic DNA (provided by R. A. Young, through the IMMLEP Program of WHO) was screened by colony hybridization using two ^{32}P-labeled overlapping 23-mer oligonucleotide probes. The recombinant λgt 11 phages (50,000 PFU/150-mm dish) were plated on *E. coli* strain Y1090 as described (1). Standard procedures for preparing nitrocellulose filter plaque replicas and carrying out hybridization reactions were used (20, 21). Recombinant plaques showing positive hybridization were isolated and purified to homogeneity. DNA inserts of all eight positive clones were analyzed by restriction mapping using the common restriction enzymes. All clones had similar restriction patterns. These recombinant clones were also examined for the expression of 10-kD protein by use of the crossreactive rabbit anti-BCG-a antibodies (gift from Dr. Percy Minden). One of the three positive clones containing 2.2-kb *M. leprae* DNA fragment was subjected to nucleotide sequencing.

DNA Sequencing. The 2.2-kb *M. leprae* DNA insert was isolated and digested with XhoI and BamHI to obtain three XhoI-BamHI fragments. These fragments were subcloned in pBluescript II KS+ phagemids (Stratagene, La Jolla, CA), and grown in DH5α host cells. The nucleotide sequence of the subcloned DNA fragments was determined by the dideoxy chain termination method, using a sequenase kit version 2.0 (U.S. Biochemical Corp., Cleveland, OH) according to the instructions provided. Both strands of the DNA were sequenced.

Expression of 10-kD Protein in E. coli. So that only the coding region of the gene encoding the *M. leprae* 10-kD protein would be expressed, a EcoRI site was engineered immediately 5′ of the start codon of the gene and a XbaI site was incorporated right after the stop codon at the 3′ end by PCR using a DNA thermal cycler and the Taq Polymerase Kit (Perkin Elmer Cetus Instruments, Norwalk, CT) according to directions of the manufacturers. The PCR fragment consisting of 320 nucleotides was digested with EcoRI, XbaI and subcloned into the same sites of the pMAL-c expression vector (generous gift from New England Biolabs, Beverly, MA). Vector containing the fused gene was transformed into the

E. coli strain PR 722 for expression by the standard procedures for DNA manipulation. In this construct the gene is cloned downstream from *mal* E gene encoding maltose binding protein (MBP). A notable feature of this vector is the presence of a sequence coding for a four-amino acid recognition site of the protease factor Xa just before the start site of the inserted gene at the 5′ end. Protein is expressed from the strong P_{tac} promoter as an MBP fusion product.

Preparation and Purification of Recombinant 10-kD Protein. Recombinant antigen was prepared in accordance with instructions provided by New England Biolabs. Briefly, cells containing the fusion plasmid were grown to log phase, and production of fusion protein was induced with 0.3 mM isopropylthiogalactoside (IPTG) at 37°C for 2 h. The pelleted bacteria were sonicated in the lysis buffer containing 0.25% Tween-20. After centrifugation at 9,000 *g*, the fusion protein was purified from the crude extract by affinity chromatography on amylose resin column. The MBP fusion protein binds to amylose. After extensive washes of the column, the fusion protein was eluted with 10 mM maltose. Subsequently, the recombinant 10-kD protein was cleaved from MBP with 1% factor Xa at room temperature for 24 h. After cleavage the 10-kD protein was separated from MBP using fast protein liquid chromatography (FPLC) (Pharmacia Fine Chemicals, Piscataway, NJ), over an anion exchange column (Mono Q) at pH 7.4 with a 0–0.3 M NaCl linear gradient.

Aliquots of the fractions were analyzed on 15% SDS-PAGE. Fractions containing purified 10-kD protein were pooled and dialyzed extensively against physiologic saline before use.

Western Blot Analysis. Electrophoresed proteins were transferred electrophoretically onto a nitrocellulose membrane. The membrane was washed with Tris-buffered saline, incubated for 1 h in 2% BSA for blocking, and incubated overnight with antibody ($1:10^3$ dilution) as described (22). The mAbs specific for the 10-kD protein of *M. tuberculosis* (SA-12, provided by Dr. Percy Minden) and specific for the protein from *M. leprae* (CS-01, supplied by B. Rivoire, Colorado State University, Fort Collins, CO) were also used. After washing, the filters were incubated with peroxidase-conjugated antiserum. Finally, the color reaction was developed with 4-chloro-1-naphthol in the presence of H_2O_2.

Assay of PBMC. PBMC (2×10^5) were cultured in 200-μl flat-bottomed wells in RPMI (Gibco Laboratories, Grand Island, NY) containing 10% AB serum, and antigen or media control. [^{3}H]Thymidine (1 μCi) was added to the cultures after 5 d, and the cells were harvested 21 h later. Assays were performed in triplicate.

IL-2 Expanded T Cell Lines and Clones. Lymphocytes were extracted from skin biopsy specimens of leprosy lesions as described (23, 24). T cell lines of cells activated in situ by *M. leprae* antigens were established by culturing in the presence of IL-2 alone. These T cell lines were used to detect the antigens that stimulate T cells in vivo. In addition, T cell clones from *M. leprae* 10-kD expanded T cell lines from peripheral blood were obtained by the method of limiting dilution (4).

T cell lines or clones, free of IL-2, were cultured in the presence of autologous irradiated feeders as APC. An assay was set up in triplicate at a density of 10^4 cloned cells per 10^5 feeders in the presence of various mycobacterial antigens. Control cultures received either no antigen, 10% IL-2 alone, or an irrelevant antigen such as tetanus toxoid. After 3 d, cultures were pulsed with [^{3}H]thymidine and harvested 4 h later.

Limiting Dilution Analysis. The precursor frequency of T lymphocytes reactive to *M. leprae* versus the native 10-kD antigen was determined by limiting-dilution analysis as previously described (25, 26).

Results

Identification of Gene Encoding the 10-kD Antigen. The immunoreactive protein determinants of *M. leprae* eliciting T cell responses were previously identified by screening proteins from *M. leprae* sonicate separated on SDS-PAGE with T cell lines/clones developed from lepromin-positive individuals stimulated in vitro with *M. leprae* or purified cell wall preparations (4). One of the two major T cell–stimulating proteins found was in the 7–10-kD molecular mass range. To characterize the proteins further, they were electroeluted from the strips of polyacrylamide gels corresponding to those in-

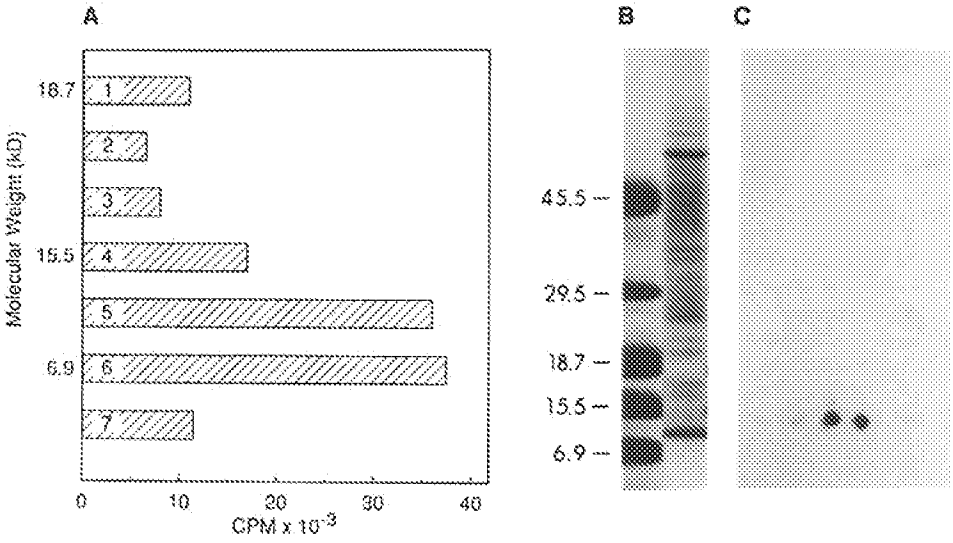

Figure 1. SDS-PAGE analysis of 7–10-kD *M. leprae* proteins. (*A*) To identify the region of the gel containing the electrophoresed *M. leprae* proteins from which the 7–10-kD T cell–stimulating antigen should be eluted, the technique of T cell Western blot was employed. This representative experiment shows the proliferative responses of a T cell line (previously reactive to a protein in the 7–10 kD molecular mass range) to proteins transferred from a portion of a gel. Proteins were eventually electroeluted from the region of the gel corresponding to the nitrocellulose strips inducing lymphoproliferation. (*B*) *M. leprae* sonicate (4 mg/ml) was subjected to electrophoresis on a 5–20% gradient SDS-PAGE. The gel was stained with coomassie blue. (*C*) T cell stimulating proteins eluted from 7–10 kD size range were resolved further by two-dimensional PAGE and stained with silver.

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                        10        20        30        40        50
M. tuberculosis 10kD    VAKVNIKPLEDKILVQANEAETTTASGLVIPDTAKEKPQEGTVVAVGPGRW
                        ||||.||||||||||||.|||| |.|||||..||||||||||||||||||
M. leprae 10kD          VAKVKIKPLEDKILVQAGEAETMTPSGLVIPENAKEKPQEGTVVAVGPGRW
                        .....|.||.|...|.  |.||....|.|....| .|.  |.|.||| ||
E. coli GroES           ---MNIRPLHDRVIVKRKEVETKSAGGIVLTGSAAAKSTRGEVLAVGNGRI

                        60        70        80        90        100
M. tuberculosis 10kD    DEDGEKRIPLDVAEGDTVIYSK-YGGTEIKYNGEEYLILSARDVLAVVSK
                        ||||.||||.||.|||.|||||.|||||||||||||||||||||||||||
M. leprae 10kD          DEDGAKRIPVDVSEGDIVIYSK-YGGTEIKYNGEEYLILSARDVLAVVSK
                         |.|. . |.||. |||||... || .. | ..|| ||.|..|.||.|..
E. coli GroES           LENGEVK-PLDVKVGDIVIFNDGYGVKSEKIDNEEVLIMSENDILAIVEA
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Figure 2. Comparison of the deduced amino acid sequences of 10-kD hsp in *M. leprae*, *M. tuberculosis* and *E. coli* (GroES). The EMBL accession number for the DNA sequence of the gene encoding the *M. leprae* 10-kD protein is X59413.

ducing proliferation of the T cell clones reactive to 7–10-kD protein (Fig. 1, *A* and *B*). The eluted material resolved into two spots on two-dimensional PAGE (Fig. 1 *C*). Proteins from an identical two-dimensional gel were transferred to an Immobilon membrane, visualized with coomassie blue stain, and subjected to protein microsequencing. The NH_2-terminal sequence of the 24 amino acids was identical to both protein spots. This amino acid sequence had striking homology to the NH_2 terminus of the *M. bovis* BCG-a protein, a 10-kD hsp differing only at residues 4, 15, and 17 of the first 20 amino acids (27–30). The sequence of the first 30 amino acid residues of the major protein of *M. leprae*, isolated by biochemical means and called the major cytosolic protein–I or the 14-kD protein, was also identical to this protein (5).

Further, by use of two overlapping 23-mer oligonucleotide probes based on the NH_2-terminal amino acid sequence, the gene encoding the 10-kD protein of *M. leprae* was isolated from the λgt 11 recombinant library of *M. leprae*. DNA inserts from all eight recombinant clones showing positive hybridization were mapped with restriction enzymes, and all had similar restriction patterns. The EcoRI insert of 2.2 kb from one of the recombinant clones was subjected to DNA sequence analysis. The sequence revealed a 300-bp coding region beginning with GTG and encoding a protein comprised of 99 amino acids, starting with alanine and with the predicted molecular mass of 10.8 kD. The deduced amino acid sequence of the 10-kD protein of *M. leprae* (Fig. 2) bears 90% identity with the 10–12-kD BCG-a antigen of *M. tuberculosis*, which was reported to have homology with hsp GroES of *E. coli* and *htp*A, a gene product of *Coxiella burnetii* (28, 29). The *M. leprae* 10-kD protein has 44% identity with GroES of *E. coli*, confirming that the 10-kD hsp are widely conserved among different organisms (31).

Our previous studies demonstrated that the 7–10-kD protein was highly effective in inducing lymphoproliferation of T cells from skin-test–positive individuals. Because only small quantities of native protein can be obtained from *M. leprae* (5), we expressed the 10-kD protein in *E. coli* to further evaluate its immunological reactivity.

Expression of Recombinant M. leprae hsp10 in E. coli. The gene encoding the *M. leprae* 10-kD antigen was expressed in *E. coli* using pMAL-*c* expression vector developed at New England Biolabs. This vector allows the foreign protein to be expressed in large amounts by fusing it to *mal* E gene encoding MBP from a strong P_{tac} promoter. Also included is a specific recognition site for the protease, factor Xa, which allows the protein of interest to be cleaved from MBP after purification on an amylose-affinity column. Fig. 3 *A* shows the SDS-PAGE analysis of both the extract of cells expressing 10-kD antigen as fusion protein and purified fusion protein before and after cleavage with factor Xa. On average, 30–40

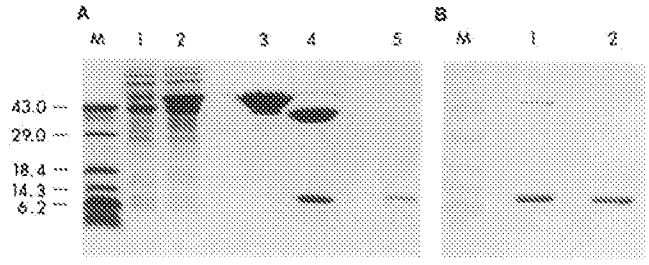

Figure 3. SDS-PAGE analysis of the recombinant 10-kD protein. (*A*) Coomassie blue–stained SDS-PAGE of *E. coli* extract containing the recombinant plasmid (pMAL-10 kD) before and after induction with IPTG (lanes *1* and *2*); purified fusion protein (lane *3*); fusion protein cleaved with protease, factor Xa, to release MBP, and the 10-kD protein (lane *4*); purified recombinant 10-kD antigen separated on FPLC after cleavage (lane *5*). (*B*) Immunoblots of recombinant MBP–10-kD fusion protein after cleavage (lane *1*); recombinant 10-kD antigen purified by FPLC on Mono Q column (lane *2*), probed with mAb CS-01 raised against the purified native 10-kD protein of *M. leprae*. This mAb does not crossreact with *M. tuberculosis* or *E. coli* hsp 10 (data not shown).

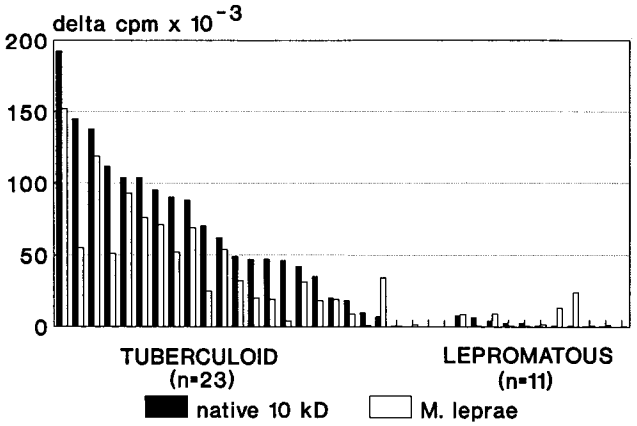

Figure 4. Comparison of PBMC responses to *M. leprae* sonicate (10 μg/ml) and the native 10-kD antigen (5 μg/ml) in patients with tuberculoid and lepromatous leprosy.

mg of fusion protein was obtained per liter of culture. After cleaving with factor Xa, the 10-kD protein was separated from MBP on an anion-exchange (Mono Q) column using FPLC. Both the fusion protein and purified recombinant 10-kD antigen reacted strongly with the mAb CS-01 (Fig. 3 *B*), as did the highly abundant native 10-kD protein of *M. leprae* against which the monoclonal was raised (data not shown).

The M. leprae 10-kD Antigen Is a Potent Stimulator of PBMC Responses. We evaluated the lymphocyte responses of patients with leprosy throughout the disease spectrum to the purified *M. leprae* hsp10 to ascertain whether the T cell response paralleled the clinical form of the disease. As shown in Fig. 4, positive PBMC responses were detected in tuberculoid patients, who have a resistant form of the disease, but negligible responses were observed in lepromatous patients, who have multibacillary disease. The magnitude of the responses seemed to parallel those to whole *M. leprae* in a large number of tuberculoid donors studied. Lepromatous donors, specifically unresponsive to *M. leprae* were also unable to respond to the 10-kD antigen; this finding suggests that T cells reactive to the 10-kD antigen were either not present in blood or unable to respond in these individuals. Therefore, the native 10-kD antigen is a strong stimulator of T cell responses in leprosy patients, and responses correlate with the clinical spectrum of disease.

T Cell Clones Recognize Specific and Crossreactive Epitopes of M. leprae 10-kD Protein. T cell clones were derived from the blood of donors who demonstrated strong in vitro responses to the 10-kD protein of *M. leprae* (Fig. 5). Two such clones were found to react to the synthetic NH_2-terminal 27-mer, one *M. leprae*-specific (Fig. 5 *A*), the other crossreactive with *M. tuberculosis* (Fig. 5 *B*). A third clone was *M. leprae* specific but recognized an epitope distal to the NH_2-terminal 27-mer (Fig. 5 *C*). The data indicate the presence of specific and crossreactive epitopes on the NH_2-terminal end of the 10-kD protein from *M. leprae*.

T cell clones that recognized the NH_2-terminal peptide of the 10-kD antigen were used as probes to determine the immunologic concordance of the native and recombinant 10-kD protein. The *E. coli* lysate containing fusion protein was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and solubilized in DMSO. The T cell clones reacted to the fusion protein present in the *E. coli* lysate as visualized by coomassie blue stain of the gel (Fig. 6 *A*). Further, the purified recombinant 10-kD antigen obtained after cleavage of fusion protein and purification on FPLC was tested for immunoreactivity. Three out of four reactive individuals showed equivalent responses to the purified recombinant 10-kD and native 10-kD protein of *M. leprae* (Fig. 6 *B*).

Comparison of T Cell Reactivity to the 10-kD Antigen Relative to Other Native and Recombinant Proteins. To assess the importance of the 10-kD antigen, in relation to other *M. leprae* proteins, in inducing peripheral blood T cell responses of lepromin-positive patients and contacts, lymphocyte proliferation to available *M. leprae* antigens was measured in vitro. As shown in Fig. 7, when peripheral blood cells from lepromin-positive donors, either patients with tuberculoid leprosy (n = 7) or healthy contacts of leprosy patients (n = 4), were tested for their responsiveness to fractions enriched for known antigenic components prepared with HPLC, highest responses were found with the 10-kD protein, followed by the 3-kD fraction, which is a mixture of several peptides (5), and the 17-kD protein. Responses to the previously identified stress protein homologs of 70, 65, and 18 kD, where present, were significantly lower. These results strongly indicate that, among the available *M. leprae* proteins,

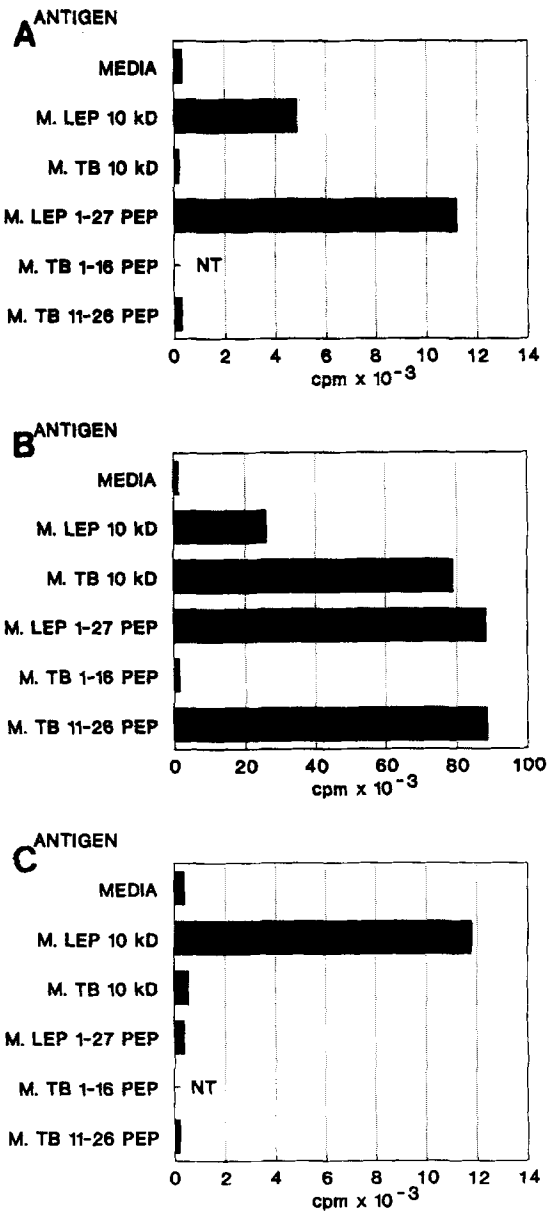

Figure 5. Patterns of T cell clone reactivity to the *M. leprae* 10-kD antigen. The results show three different reactivity patterns based on epitope recognition. Clones were tested against native 10-kD antigens of *M. leprae* (5 μg/ml) and *M. tuberculosis* (5 μg/ml) and synthetic peptides (10 μg/ml). (*A*) An *M. leprae*-specific NH_2-terminal peptide–reactive T cell clone. (*B*) An *M. leprae–M. tuberculosis* crossreactive, NH_2-terminal peptide–reactive T cell clone. (*C*) An *M. leprae*-specific, NH_2-terminal peptide–nonreactive T cell clone. NT, not tested.

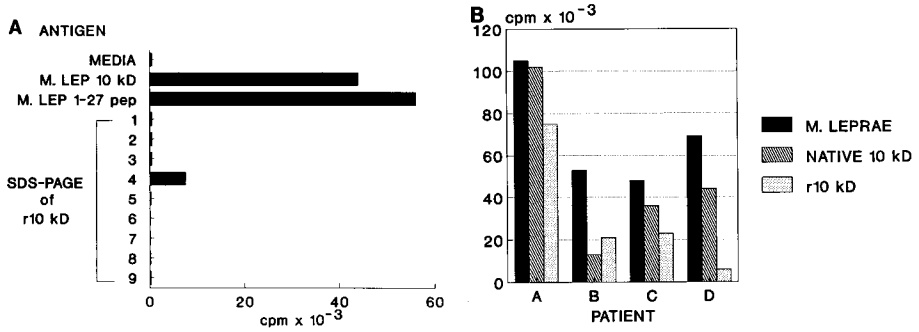

Figure 6. T cell responses to the native and recombinant 10-kD antigen. *M. leprae* sonicate was tested at 10 μg/ml, the native 10-kD antigen at 5 μg/ml, and the recombinant 10-kD antigen at 1 μg/ml. (*A*) A T cell clone reactive to the native 10-kD antigen and the NH_2-terminal peptide identifies the recombinant MBP–10-kD fusion protein on a T cell Western blot. (*B*) PBMC responses in four tuberculoid patients to the native and recombinant 10-kD antigen.

the 10-kD protein appears to be most potent in stimulating T cells; with in vitro responses that were comparable to those elicited by whole mycobacteria.

Precursor Frequency Analysis of 10-kD-reactive T Cells. For comparison of the precursor frequencies of 10-kD– and *M. leprae* sonicate–reactive T cells, limiting-dilution analysis was performed on peripheral blood T cells from a skin-test–positive patient contact and a patient with tuberculoid leprosy. Fig. 8 shows the results from the patient contact. In this donor 1/768 T cell precursors were reactive to whole *M. leprae* sonicate, and 1/2,191 were reactive to the native 10-kD antigen. Similar results were obtained in the study of the tuberculoid patient; 1/400 precursors were reactive to the *M. leprae* sonicate as compared with 1/1,150 that were reactive to the 10-kD antigen. Therefore, in both subjects, about one third of the *M. leprae*–reactive T cells seem to be responding to the 10-kD protein.

Potential Use of M. leprae 10-kD Antigen as a Skin-test Reagent. When T cells were derived from tuberculoid lesions and cultured in the presence of IL-2 alone to expand those cells activated in vivo to express IL-2 receptors, reactivity was found to *M. leprae* cell walls. The model for cutaneous DTH in leprosy is the 3-wk granulomatous response to intradermal challenge with *M. leprae*, called the Mitsuda skin-test reaction (18). Therefore, we derived T cells from Mitsuda reactions and expanded these cells in the presence of IL-2 for 1–2 wk without antigen. These T cell lines were found to

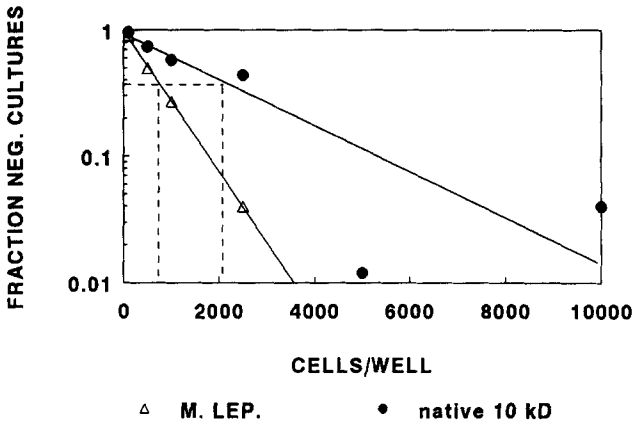

Figure 8. Precursor frequency analysis by limiting dilution in a lepromin-positive patient contact. *M. leprae* sonicate was used at 10 μg/ml, the native-10 kD antigen at 5 μg/ml. The results show the number of T cell precursors capable of responding to a given antigen.

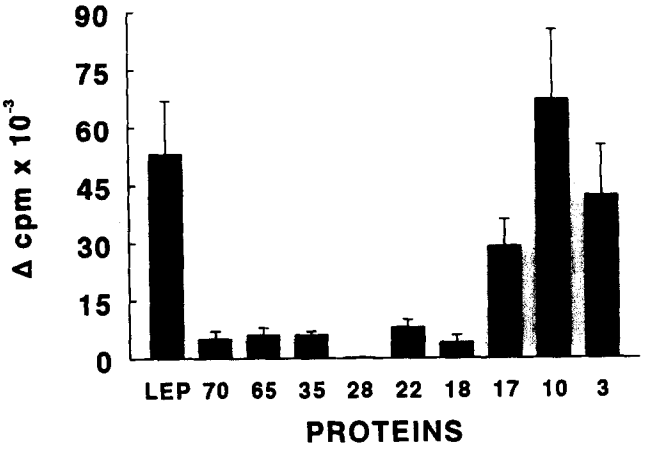

Figure 7. PBMC responses of *M. leprae*–reactive donors to purified native and recombinant mycobacterial proteins. All antigens were tested at 10 μg/ml, except the 10-kD antigen, which was tested at 5 μg/ml. The 70-, 65-, and 18-kD antigens are recombinant proteins, the 35-, 28-, 22-, 17-, 10-, and 3-kD antigens are native proteins. LEP, *M. leprae*.

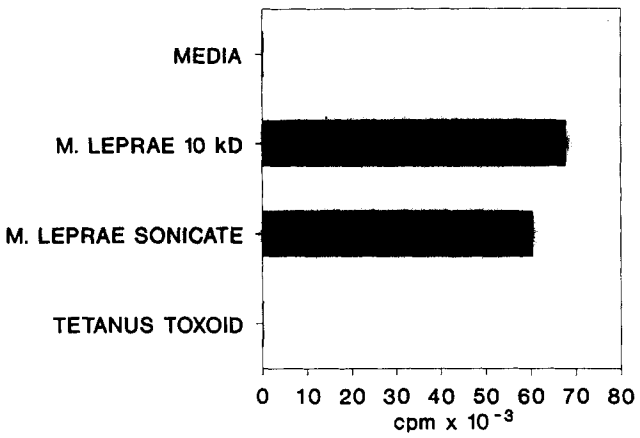

Figure 9. Reactivity of a T cell line derived from a lepromin skin test (Mitsuda reaction) to the native 10-kD antigen. The T cells extracted from the skin biopsy specimen were cultured in the presence of IL-2 to expand those cells stimulated by antigen in vivo. The results are shown for one representative patient; four patients were studied.

proliferate equally well in response to the 10-kD antigen as to whole *M. leprae*, but not to the unrelated antigen. This observation indicates that the responsive T cell component fundamental to the cutaneous measure of DTH in leprosy is the 10-kD reactive T cell (Fig. 9).

Although the 10-kD antigen of *M. leprae* demonstrated induction of T cell reactivity in vitro, in vivo studies were required to assess the importance of this antigen for inducing cell-mediated immunity. Thus, to examine the DTH reactive in vivo, guinea pigs sensitized to *M. leprae* were tested 1–2 mo later with intact *M. leprae* and with recombinant 10-kD antigen. Fig. 10 shows that the 10-kD antigen was as effective as *M. leprae* in eliciting DTH responses in these immunized animals.

Discussion

The mechanism by which the host responds to and eliminates infection is a central issue in our understanding of the immune response to pathogens. For many intracellular organisms, such as *M. leprae*, the T cell response is crucial. Our data provide evidence that the 10-kD antigen of *M. leprae* is a strong stimulator of T cell responses: (*a*) The *M. leprae* 10-kD protein elicited PBMC responses similar in magnitude to the response to *M. leprae* bacillus, and these responses paralleled the clinical and immunologic spectrum of disease. (*b*) The 10-kD protein evoked greater PBMC responses than other purified and recombinant antigens. (*c*) Limiting-dilution analysis performed in two *M. leprae*–reactive individuals indicated that approximately one third of *M. leprae*–reactive T cell precursors responded to the *M. leprae* 10-kD antigen. (*d*) T cells derived from a lepromin skin test (Mitsuda reaction) showed marked proliferation in response to the *M. leprae* 10-kD antigen. (*e*) Like intact *M. leprae*, the *M. leprae* 10-kD antigen elicted DTH responses in sensitized guinea pigs. (*f*) T cell clones recognized specific and cross-reactive epitopes on the *M. leprae* 10-kD antigen. (*g*) T cells responded similarly to the native and recombinant forms of this antigen.

An important finding of the present study was that the *M. leprae* 10-kD protein, a major T cell antigen, shares significant homology with GroES of *E. coli* (31). In *E. coli*, the *Gro*ES gene is contained in a single *Gro*E operon encoding another stress protein, GroEL, with which it forms a heterodimer containing six to eight GroES subunits and 14 GroEL subunits forming a complex composed of two stacked rings each containing seven subunits of GroEL (31). The GroEL and GroES are reported to be among the most abundant proteins in the cell. The hsp10 is an essential gene in *E. coli* and yeast, and it functions as a chaperonin enabling transport and

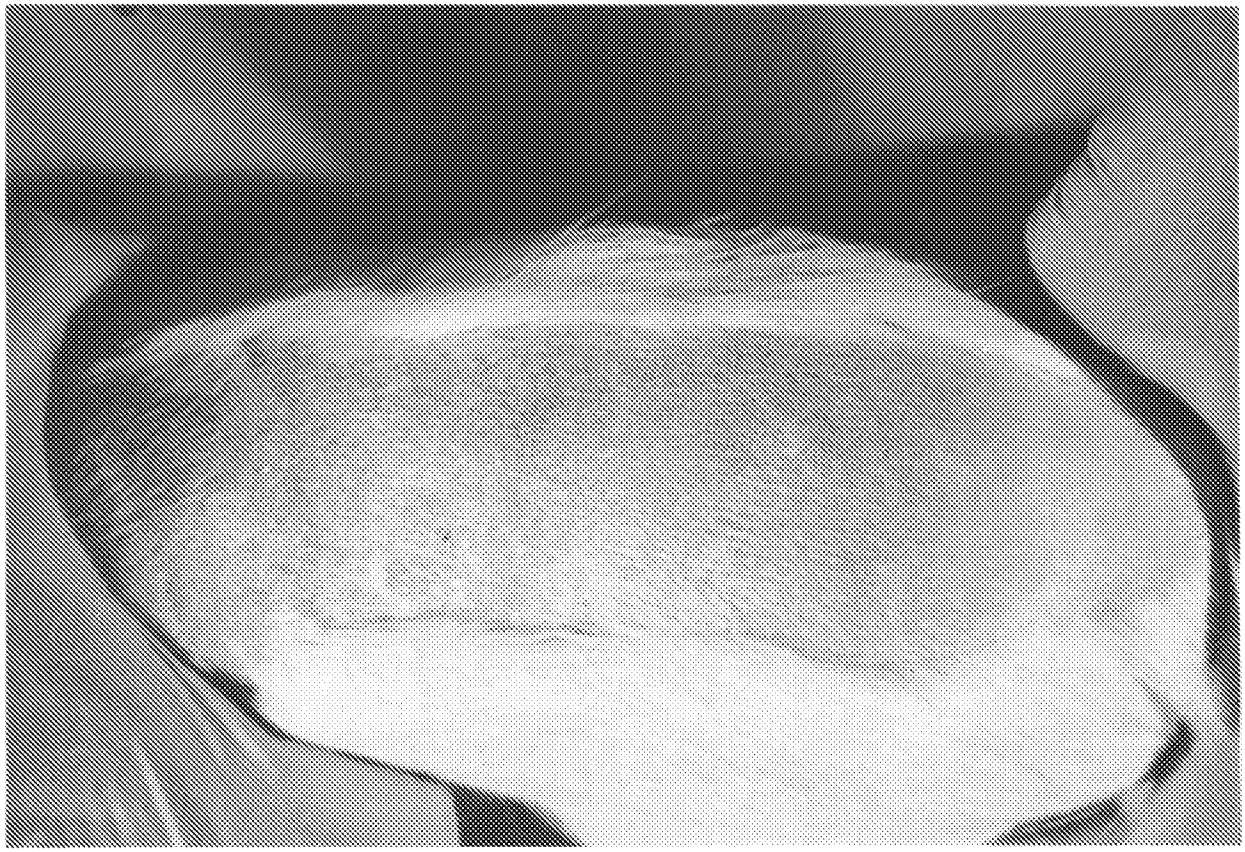

Figure 10. DTH reactions elicted by recombinant 10-kD antigen. Guinea pigs immunized with 500 μg of killed *M. leprae* in saline were skin tested 1 mo later with (*1*) 10 μg/0.1 ml *M. leprae*; (*2*) 0.7 μg, (*3*) 2.2 μg, (*4*) 6.6 μg, and (*5*) 20.0 μg/0.1 ml of recombinant 10-kD antigen, injected intradermally. The orientation of the skin reactions is as follows: [1 3 5 / 2 4]

folding of molecules across membranes (31, 32). While its function in mycobacteria is unknown, its expression in both cytosol and purified cell walls is consistent with its functioning as a chaperonin.

Since stress proteins are highly conserved across many genera, the ability of an immune host to recognize these proteins as antigens raises fundamental questions about the regulation of the immune response (33). On the one hand, the ability of the immune system to recognize stress proteins provides the ability to react with the wide variety of pathogens that express these antigens. Therefore, if hsp are involved in providing protective immunity to one pathogen, the highly conserved nature of heat-shock cognates could result in some level of crossresistance to another. Such a hypothesis has been invoked to explain the function of γ/δ T cells, which in certain experimental systems appear to recognize the mycobacterial 65-kD hsp (34). Furthermore, the ability of the host to recognize cognate autologous stress proteins would facilitate recognition and disposal of an infected, hence stressed, host cell (35). Of course, if the immune response is directed against species-specific epitopes, this kind of crossreactive immunity would not be seen. On the other hand, the crossreactive nature of stress proteins may, in some MHC types, inappropriately result in the autoimmune responses, for example, as implicated by the response of patients with rheumatoid arthritis to the 65-kD antigen (36). The regulatory mechanisms by which the immune system can direct a strong response to a bacterial stress protein, yet not destroy self, are not well understood. The identification of the *M. leprae* GroES homologue as a major T cell stimulating antigen provides an opportunity to study the regulation of the immune response to such stress proteins.

The identification of the *M. leprae* 10-kD protein as a major stimulator of the T cell response in immune and resistant individuals has certain implications for control of leprosy. Leprosy affects several million people worldwide and is a tremendous health and economic burden on developing countries. A key issue is the identification of infected individuals who may be incubating the disease and may be at risk for spreading the disease to susceptible individuals. The development of a sensitive skin-test reagent with specificity for *M. leprae* infection, analogous to the purified protein derivative (PPD) used to assess infection by *M. tuberculosis*, would help in early detection of infection. Lepromin, prepared from infected multibacillary human or infected armadillo tissues, is the current standard (37). However, the lack of uniformity and the high cost of lepromin significantly hamper epidemiologic research as well as leprosy control (38). Since our findings suggested that a major T cell component of lepromin reactivity in vitro was directed against the 10-kD antigen, the purified recombinant protein was tested in vivo in *M. leprae*–sensitized guinea pigs and was found to be a very strong skin-test antigen. Clinical studies are underway to ascertain whether it can discriminate between lepromin-positive and lepromin-negative patients with leprosy.

The use of multiple drug therapy has recently affected the transmission of leprosy (39). Yet the side effects of these drugs as well as the difficulty and cost of administering them have stimulated research toward development of vaccines against leprosy. It is now feasible to generate subunit or live recombinant vaccines by use of several adjuvant or vector systems. Since BCG was found to have some degree of protective efficacy against leprosy in early trial studies, one such vaccine vehicle is obviously recombinant BCG, and it would be possible to express any *M. leprae* antigen thought to be required for protection in rBCG (40–42). The fundamental challenge remains the identification of *M. leprae* antigens required for protection.

In summary, the present data indicate that the 10-kD antigen of *M. leprae* is a major T cell antigen, and it may have use in the immunoprophylaxis of leprosy. Further studies are needed to determine its role in inducing protective immunity and determining the mechanism whereby a host reacts to stress antigens of pathogens, but yet is prevented from reacting to the homologous proteins in the "self." The present results provide a framework for addressing such issues.

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